

# Biosensors Based on Cross-linking of Biotinylated Glucose Oxidase by Avidin

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## **Abstract**

Avidin, having four biotin binding units, cross-links and immobilizes glucose oxidase (GOX) labeled with multiple biotins (B-GOX) when solutions of B-GOX and avidin are mixed on an electrode. The  $\text{H}_2\text{O}_2$  flux generated in the B-GOX catalyzed oxidation of glucose by dissolved  $\text{O}_2$  is measured as an electroreduction current at a horseradish peroxidase (HRP) redox conducting hydrogel electrode poised at +100mV (Ag/AgCl). The sensitivity of the resulting glucose sensor is  $0.14\text{A cm}^{-2} \text{M}^{-1}$  with a linear range up to 2mM glucose.

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Avidin, having four biotin binding units, cross-links and immobilizes glucose oxidase (GOX) labeled with multiple biotins (B-GOX) when solutions of B-GOX and avidin are mixed on an electrode. The H<sub>2</sub>O<sub>2</sub> flux generated in the B-GOX catalyzed oxidation of glucose by dissolved O<sub>2</sub> is measured as an electroreduction current at a horseradish peroxidase (HRP) redox conducting hydrogel electrode poised at +100mV (Ag/AgCl). The sensitivity of the resulting glucose sensor is 0.14A cm<sup>-2</sup> M<sup>-1</sup> with a linear range up to 2mM glucose.

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## Introduction

Electrochemical biosensors [1,2,3], reactors [2,4], and optical biosensors [3,5] utilize immobilized enzymes. Usually immobilization involves a covalent bond forming reaction of the enzyme, e.g. with glutaraldehyde or with diepoxide. Sensitive  $\text{H}_2\text{O}_2$  electrodes, built by the covalent immobilization of horseradish peroxidase (HRP) in an electron conducting redox hydrogel that electrically connected redox centers of bound HRP to vitreous carbon electrodes, have been previously described [6,7]. The sensitivity of these electrodes was remarkably high ( $1\text{A cm}^{-2}\text{ M}^{-1}$ ). We then followed Kulys [8,9,10], Gorton [11,12], and Tatsuma and Watanabe [13] who coupled sensitive electrochemical  $\text{H}_2\text{O}_2$  detection with oxidase catalyzed  $\text{H}_2\text{O}_2$  production, forming biosensors sensitive to specific oxidase substrates. Specifically, we built a series of single and bilayer (inner layer HRP and outer layer oxidase) bienzyme electrochemical biosensors sensitive to glucose, lactate, choline, methanol, ethanol, and D-amino acids [14].

While we immobilized the enzymes by covalent bonding, Bourdillon et al [15] immobilized GOX at an electrode surface using antibody/antigen bioconjugates. Using the bioconjugate modified surface Bourdillon et al precisely characterized the electrochemical kinetics and charge transport in monolayer enzyme electrodes. Because the biotin-avidin complex has a dissociation constant of  $\sim 10^{-15}$  [16], this conjugate is frequently used for immobilization of reagents on surfaces [17,18] including surfaces of electrodes [19,20]. Otto et al. [21] reported the binding of a GOX-avidin layer to a biotin containing phospholipid membrane and the forming of a glucose biosensor with improved stability. We reported direct electrical detection

of the occurrence of the avidin-biotin affinity reaction based on conjugation of biotin labeled horseradish peroxidase (B-HRP) to avidin in an electron conducting redox hydrogel. Specifically, we measured the  $\text{H}_2\text{O}_2$  electroreduction current at +100mV SCE that flowed when B-HRP contacted the "wired" avidin on the electrode.

Here (Figure 1a) we react, on the surface of an HRP containing electron conducting redox hydrogel coated electrode, B-GOX with multiple biotin functions with avidin that binds 4 biotin molecules, to form a well cross-linked B-GOX rich layer. The glucose and oxygen flux are detected by production of  $\text{H}_2\text{O}_2$  in the outer layer and  $\text{H}_2\text{O}_2$  electroreduction to water in the inner layer. We also immobilize avidin on top of the HRP containing redox hydrogel layer by cross-linking the avidin with a diepoxide. Binding of B-GOX to the avidin rich film also produces a B-GOX layer on the electron conducting HRP redox polymer layer.

## Experimental Section

**Reagents.** Avidin (#A-9275), biotin (#B-4501), horseradish peroxidase (#P-6782), glucose oxidase (#G-7141), and biotin labeled glucose oxidase with 5.1 biotins per GOX (#G-3636) were purchased from Sigma. Sigma reports the activity of B-GOX as 200 units/mg, where one unit of B-GOX oxidizes 1.0  $\mu$ mole of  $\beta$ -D-glucose to D-gluconic acid and  $H_2O_2$  per min. at pH 5.1 at 35°C, and the activity of HRP as 280 units/mg, where one unit of HRP forms 1.0 mg of purpurogallin from pyrogallol in 20 sec at pH 6.0 at 20°C. Poly(ethylene glycol 400 diglycidyl ether) (PEGDGE), tech. grade was purchased from Polysciences (#08210). A 30% hydrogen peroxide solution was purchased from Aldrich, and its concentration was verified by measuring its density [22]. The redox polymer (PVP-NH<sub>2</sub>-Os) was synthesized from a poly(4-vinylpyridine) backbone by partially complexing with Os(bpy)<sub>2</sub>Cl<sup>+/2+</sup> and partial quaternization with 2-bromoethylamine. The osmium complexes allowed redox conduction between the glassy carbon electrode and HRP heme centers while the primary amine modification provided amines for cross-linking. The ratio of unmodified pyridines to osmium complexed to bromoethylamine quaternized was 1 : 3.35 : 0.6. Preparation of this redox polymer (PVP-NH<sub>2</sub>-Os) has been described [23]. All other chemicals were of the best available commercial grade, purchased from Aldrich or Sigma and used as received.

**Solutions, Electrodes and Electrochemical Equipment.** Hydrogen peroxide and enzyme solutions were prepared daily. The glucose solution (1M) was allowed to mutarotate before use. All solutions of B-GOX, biotin and avidin were prepared and electrochemical measurements were performed in a modified

Dulbecco's buffer (PBS) pH 7.4. 3mm vitreous carbon rotating disk electrodes were built as previously described [6]. Ring/disk electrodes 5mm dia. glassy carbon disk and platinum ring were purchased from Pine Instruments (#AFMRT28TGCPT). The electrodes were polished successively with 5 $\mu$ m, 1 $\mu$ m and 0.3 $\mu$ m alumina with thorough sonication after each polishing step. A Pine Instruments rotator AFMSRX coupled with an ACMDI 1906C shaft was used for the disk electrodes and a ACMDRD 2805 shaft was used for the ring/disk electrodes. The electrochemical measurements were performed in a standard three electrode cell with a platinum foil counter and a Ag/AgCl Bioanalytical Systems reference electrode. All potentials are reported relative to that of the Ag/AgCl reference electrode. An EG & G potentiostat/galvanostat Model 173 was used to take the electrochemical measurements. The volume of the cell was 125mL. It contained, in all experiments, 100mL of PBS.

**Enzyme Immobilization.** The films for the ring/disk experiments were made by mixing a 5mg/mL avidin solution and a 5mg/mL B-GOX solution at different ratios and applying a measured volume to the 5mm dia. disk electrode. After drying, the films were cured for a minimum of 24 hours at room temperature before use. We first applied a H<sub>2</sub>O<sub>2</sub> sensing layer comprising HRP and PVP-NH<sub>2</sub>-Os polymer on the 3mm vitreous carbon electrodes as previously described [6]. After curing this layer for 48 hours, we applied a GOX containing second layer by either of two methods. 1) Coating a layer of B-GOX/avidin, made by mixing a 5mg/mL B-GOX solution and a 5mg/mL avidin solution at a 5:4 volume ratio (B-GOX:avidin), loading different amounts of the mixture onto the electrode, drying

and curing for > 48 hours at room temperature (Figure 1a). 2) Coating a layer of avidin on the HRP layer, made by mixing a 10mg/mL avidin solution and a 6mg/mL PEGDGE solution in a 6:1 volume ratio (avidin:PEGDGE) and loading 2 $\mu$ L. After drying, the electrode was cured for > 48 hours at room temperature before the next stage, where a GOX layer was allowed to self-assemble by rotating the electrode in a 100 $\mu$ g/mL B-GOX solution for 20 min. The B-GOX permeated the film complexing the avidin (Figure 1b). The electrodes were thoroughly washed with PBS buffer for 15 min. and were used promptly for the measurements.

**Measurements.** The disc or ring/disk electrodes were rotated at 1000 rpm. The disk electrodes were poised at +100mV vs. Ag/AgCl. With the ring/disk electrodes the H<sub>2</sub>O<sub>2</sub> detecting platinum ring was poised at +700mV, and the disk was left to float at solution potential, i.e. was left at open circuit. H<sub>2</sub>O<sub>2</sub> or glucose was added as aliquots of concentrated stock solutions so as to hold the 100mL solution volume reasonably constant.

## **Results and Discussion**

**Ring/disk experiments.** The production of H<sub>2</sub>O<sub>2</sub> by B-GOX immobilized by the cross-linked avidin on the disk of the ring disk electrode (Figure 2) was first measured by its electro-oxidation on the platinum ring at +0.7V. Figure 3 shows the response to glucose of a series of ring/disk electrodes differing in the weight % of avidin in their films at a constant total loading of 460 $\mu$ g/cm<sup>2</sup>. The H<sub>2</sub>O<sub>2</sub>



electroreduction current increased linearly from 0.01mM to 1mM glucose reaching a plateau at  $\approx 1.4$  mM. Sensitivity and the plateau depended on the avidin content.

Figure 4 shows the dependence of the ring current at 0.71mM glucose concentration on the avidin wt.% in the films. A maximum is observed at 44 wt.% avidin.

Figure 5 shows the ring currents for different loadings of an avidin/B-GOX (44 wt.%/56 wt.%) film. The current increased linearly from 0.01mM to 4mM glucose for the thinnest film ( $25\mu\text{g}/\text{cm}^2$ ) and from 0.01mM to 1mM for the thickest film ( $1275\mu\text{g}/\text{cm}^2$ ). Thicker films containing more B-GOX consume more  $\text{O}_2$  and produce more  $\text{H}_2\text{O}_2$ , but when the  $\text{O}_2$ , the concentration of which is only  $2.4 \times 10^{-4}$  M in air saturated water, is depleted the glucose response becomes non-linear. The current at saturating glucose concentrations increased with film thickness.

Figure 6 shows the increase in the ring current at 0.71mM glucose concentration with the thickness of the 44/56 B-GOX-avidin film. At a loading of  $255\mu\text{g}/\text{cm}^2$ , the current reached 86% of the current of the thickest film electrode ( $1275\mu\text{g}/\text{cm}^2$ ). Up to about  $250\mu\text{g}/\text{cm}^2$  the sensitivity increases linearly with loading, i.e. film thickness.

**Bilayer bienzyme sensor.** Figure 7 shows the  $\text{H}_2\text{O}_2$  electroreduction current's dependence on the glucose concentration in bienzyme disk electrodes with an inner HRP and an outer B-GOX/avidin layer. The avidin/B-GOX weight ratio in the

outer layer was 4:5, because at this 4:5 ratio the sensitivity was highest. At a loading of less than  $25\mu\text{g}/\text{cm}^2$  of avidin/B-GOX, the current increased linearly from 0.01mM to 2mM glucose, while at a loading exceeding  $25\mu\text{g}/\text{cm}^2$  the current increased linearly from 0.01mM to only 1mM glucose. Increasing loading of avidin immobilized B-GOX resulted in higher sensitivity (Figure 8). However, at high glucose concentration, thinner film electrodes ( $< 25\mu\text{g}/\text{cm}^2$ ) saturated and response plateaued, while the current response in thicker film electrodes ( $>25\mu\text{g}/\text{cm}^2$ ) reached a maximum then declined slowly before plateauing. This last effect was particularly pronounced in the thickest electrodes where the current loss was 31% (for a loading of  $255\mu\text{g}/\text{cm}^2$ ) between 2.2mM (maximum response) and 22mM (plateau region).

This phenomenon is explained by competition of the  $\text{Os}^{+3}$  centers of the redox polymer (in the  $\text{H}_2\text{O}_2$  sensing layer) with dissolved  $\text{O}_2$  for the GOX  $\text{FADH}_2$  electrons (Figure 9). Indeed, when a very thick layer of GOX is exposed to high glucose concentrations, oxygen is rapidly depleted in the upper layers of the film. At the same time production of  $\text{H}_2\text{O}_2$  reaches its maximum causing a substantial fraction of the osmium in the  $\text{H}_2\text{O}_2$  sensing layer to reside in the +3 state. In the lower B-GOX layers of the now  $\text{O}_2$  depleted thick films the GOX  $\text{FADH}_2$  centers are oxidized mostly by  $\text{Os}^{+3}$  centers in the polymer film. This oxidation is primarily at the interface of the two layers and depends on their mixing. Thus the net decrease in the  $\text{H}_2\text{O}_2$  electroreduction current is caused by "shorting" of  $\text{FADH}_2$  sites to the electrode by the redox polymer.

**Comparison of the sensitivity between ring/disk and bienzyme electrodes.** The sensitivity of the bienzyme electrode is higher than that of the ring/disk electrode. For example, at 0.71mM glucose concentration and with a  $25\mu\text{g}/\text{cm}^2$  B-GOX/avidin loading on both electrodes, the sensitivity of the bienzyme disks was  $40.5\mu\text{A}/\text{cm}^2$  and that of the GOX disk/Pt-ring system was only  $0.92\mu\text{A}/\text{cm}^2$ , a 44 fold difference. Since both generate about the same amount of  $\text{H}_2\text{O}_2/\text{cm}^2$ , the  $\text{H}_2\text{O}_2$  collection efficiency of a bienzyme electrode is, as one would expect, higher than that of the ring/disk electrode. The higher collection efficiency is related to not only the well recognized collection constant of ring/disk systems, i.e. ring-disk, but also to the 2-3 fold higher sensitivity of the HRP electrodes relative to that of the platinum electrode.

**Anodic Glucose Response.** When poised at +550mV, glucose is electro-oxidized on the bienzyme bilayer electrodes because part of their GOX is "wired" to the electrode by the redox polymer. Under an argon atmosphere, the glucose response measures the extent of the previously discussed short circuiting. Under argon, no trend was noted for the sensitivity or plateau when the GOX film thickness was varied, showing that the anodic current is generated at the interface between the HRP and GOX layers and not in the GOX layer itself. However, under air the anodic current at saturating (50mM) glucose concentration declined at higher B-GOX loadings, i.e. thicker avidin bonded films (Figure 10), because  $\text{O}_2$  competed with  $\text{Os}^{+3}$  for the  $\text{FADH}_2$  sites.  $\text{H}_2\text{O}_2$  generated in the  $\text{O}_2$  reaction then oxidized the  $\text{Os}^{+2}$  to  $\text{Os}^{+3}$  in the inner "wired"  $\text{H}_2\text{O}_2$  sensing layer. This creates a short circuit similar to that seen in thick film electrodes operated at +100mV, where

a cathodic current from B-GOX generated  $\text{H}_2\text{O}_2$  is detected. The thicker layers of GOX show a greater propensity for short circuiting because they produce more  $\text{H}_2\text{O}_2$ .

**$\text{H}_2\text{O}_2$  Response.** Using the same series of bienzyme electrodes operated at +100mV, we measured hydrogen peroxide response instead of glucose response (Figure 11). The current increased linearly from  $1\mu\text{M}$  to  $400\mu\text{M}$   $\text{H}_2\text{O}_2$  and then reached a plateau. The linear range was independent of B-GOX/avidin film thickness. However, the sensitivity was affected by the thickness of the B-GOX layer. Figure 12 shows the  $\text{H}_2\text{O}_2$  response at  $41\mu\text{M}$   $\text{H}_2\text{O}_2$  (i.e. in the linear range) when the B-GOX thickness was increased. For loadings between  $5\mu\text{g}/\text{cm}^2$  and  $\sim 120\mu\text{g}/\text{cm}^2$  a  $3.7\mu\text{A}$  plateau was observed. This meant that  $\text{H}_2\text{O}_2$  diffusion through the B-GOX/avidin film was not limiting. However, for a loading  $>120\mu\text{g}/\text{cm}^2$ , the current decreased with increasing film thickness. At  $510\mu\text{g}/\text{cm}^2$  loading, the current only reached  $1.87\mu\text{A}$ , i.e. half of the plateau.

**Comparison of the  $\text{H}_2\text{O}_2$  and glucose response of the bienzyme sensor.** At  $0.71\text{mM}$  glucose concentration, i.e. in the linear range, the glucose sensitivity increased with the thickness of the film (Figure 8) while the hydrogen peroxide sensitivity decreased (Figure 12). For loadings  $>120\mu\text{g}/\text{cm}^2$  B-GOX/avidin, the  $\text{H}_2\text{O}_2$  diffusion through the film began being impeded, and the  $\text{H}_2\text{O}_2$  sensitivity started to decline. Glucose sensitivity in the linear range always increased between  $5.1\mu\text{g}/\text{cm}^2$  and  $510\mu\text{g}/\text{cm}^2$  loadings. However, the  $\text{H}_2\text{O}_2$  sensitivity was always higher than the glucose sensitivity even for the thickest B-

GOX films ( $510\mu\text{g}/\text{cm}^2$ ). For a  $12.7\mu\text{g}/\text{cm}^2$  B-GOX film the  $\text{H}_2\text{O}_2$  current response was  $3.76\mu\text{A}$  at  $41\mu\text{M}$   $\text{H}_2\text{O}_2$ . The same current was obtained for a glucose concentration of  $2\text{mM}$ . This meant that  $2\text{mM}$  of glucose produced an equivalent  $\text{H}_2\text{O}_2$  concentration of  $41\mu\text{M}$ . Ideally, we could make a system where the electrodes' sensitivities to  $\text{H}_2\text{O}_2$  and glucose approached each other. Practically, we are limited by the escape of  $\text{H}_2\text{O}_2$  generated in the B-GOX layer to the solution and by the short circuiting reactions.

**Control experiments.** In control experiments the B-GOX was replaced by GOX and biotin was added to compete with B-GOX for avidin sites in the B-GOX binding reaction (Figure 13). When avidin was cross-linked with B-GOX, the sensitivity and plateau were higher than in the GOX and B-GOX + biotin controls. Evidently, non-biotinylated GOX was not cross-linked and immobilized by avidin, and the free biotin reduced the B-GOX binding sites. However, a significant current was still observed in the control systems, showing non-specific adsorption of the negatively charged GOX and B-GOX on the positively charged avidin and inner  $\text{H}_2\text{O}_2$  sensing layer.

**Self-assembled bienzyme sensor.** Figure 14 shows the response to glucose for 2 bienzyme (i.e. HRP and GOX) electrodes made by self-assembling. Incubation of the avidin loaded electrode with B-GOX binds solution B-GOX and immobilizes it. The current response is similar to that of a bienzyme electrode made with a  $5.1\mu\text{g}/\text{cm}^2$  film of B-GOX cross-linked with avidin (see Figure 7). To accommodate this amount of B-GOX on the electrode several layers of B-GOX

must be immobilized in the film. This indicates that binding sites both at the solution interface and within the avidin film are accessible to B-GOX. When the same electrode is pretreated with biotin, all the binding sites of the avidin layer are occupied, and a much lower current is observed. Although ideally no current should have been observed in the biotin treated electrodes, the current decreased only by an order of magnitude. The residual current is explained by non-specific adsorption of negatively charged GOX by positively charged avidin at pH 7.4. Indeed, when the same experiments, i.e. both incubation steps and measurement of glucose response, were carried out in a 1M NaCl buffer, the glucose response was identical for the electrode untreated with biotin but ~5000 times smaller for the biotin treated electrode. The non-specific adsorption phenomenon was reduced by a factor of ~500.

### **Conclusion**

It is shown that a multiply biotin derivatized enzyme can be cross-linked with avidin, and that enzyme-based sensors can be made by spontaneous immobilization of biotinylated enzymes with avidin.

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### Figure Captions


Figure 1. Schematic drawing of bilayer-bienzyme sensors on vitreous carbon electrodes with an electrically wired HRP inner layer and a nonwired outer layer of B-GOX either cross-linked with avidin (a) or self-assembled in a previously immobilized avidin layer (b). (X): avidin, (<>): biotin, (): B-GOX.


Figure 2. Schematic drawing of the ring/disk electrode coated with a B-GOX/avidin film. Disk at open circuit and ring at +700mV Ag/AgCl. (X): avidin, (<>): biotin, (): B-GOX.

Figure 3. Dependence of the glucose produced current of ring/disk electrodes on the avidin/B-GOX ratio. H<sub>2</sub>O<sub>2</sub> formed by the action of B-GOX on glucose at the disk is measured at the ring electrode. Disk films were made by varying the amount of avidin in the film while keeping the total loading constant (460μg/cm<sup>2</sup>). Conditions: 1000rpm, PBS pH= 7.4, air, platinum ring poised at +700mV, disk left at open circuit.

Figure 4. Dependence of the glucose current sensitivity on the avidin weight fraction for ring/disk electrodes prepared as in fig. 3. Conditions: 0.71mM glucose (linear range), 1000rpm, PBS pH= 7.4, air, ring poised at +700mV, disk left at open circuit.

Figure 5. Dependence of the glucose produced current of ring/disk electrodes on the B-GOX/avidin film thickness. Ring/disk electrodes were prepared with a fixed

44% avidin / 56% B-GOX ratio in the films on the disk. Conditions: 1000rpm, PBS pH= 7.4, air, ring poised at +700mV, disk left at open circuit.

Figure 6. Dependence of the glucose current sensitivity on the B-GOX film thickness at 0.71mM glucose concentration (linear range). Ring/disk electrodes were prepared as in Fig. 5. Conditions: 1000rpm, PBS pH= 7.4, air, ring poised at +700mV, disk left at open circuit.

Figure 7. Dependence of the cathodic glucose current on the outer B-GOX film thickness for bilayer bienzyme electrodes.  $H_2O_2$  formed by the catalytic action of B-GOX on glucose is detected at the inner HRP electrode by a cathodic current response. Electrodes were prepared with optimized 44% avidin / 56% B-GOX films at different loadings. Conditions: 1000rpm, PBS pH= 7.4, air, +100mV.

Figure 8. Dependence of the glucose current sensitivity on the B-GOX film thickness at 0.71mM glucose concentration for bilayer-bienzyme electrodes. Electrodes prepared as in fig. 7. Conditions: 1000rpm, PBS pH= 7.4, air, +100mV.

Figure 9. Redox cycles for bilayer-bienzyme electrodes. Path 1: Desired behavior for the system showing the electrocatalytic reduction on a wired HRP electrode of  $H_2O_2$  produced by the B-GOX. Path 2: System short circuiting where  $Os^{3+}$  centers generated by HRP catalytic reduction of  $H_2O_2$  in the inner HRP layer compete with oxygen to reoxidize reduced GOX flavins in the outer B-GOX layer.



Figure 10. Percentage loss in anodic glucose current response for bilayer-bienzyme electrode at 50mM glucose concentration with different B-GOX/avidin film loadings when the argon atmosphere is switched to air. Electrodes prepared as in fig. 7. Conditions: 1000rpm, PBS pH= 7.4, +550mV.

Figure 11. Dependence of the cathodic H<sub>2</sub>O<sub>2</sub> current response on the thickness of the outer B-GOX/avidin layer. Electrodes prepared as in fig. 7. Conditions: 1000rpm, PBS pH= 7.4, air, +100mV.

Figure 12. Dependence of H<sub>2</sub>O<sub>2</sub> sensitivity on the B-GOX film thickness at 41μM H<sub>2</sub>O<sub>2</sub> concentration (linear range). Electrodes prepared as in Fig. 7. Conditions: 1000rpm, 20mM phosphate, pH= 7.4, air, +100mV.

Figure 13. Control experiments showing the necessity of effective avidin cross-linking. ( ▲ ) Electrode made with the standard B-GOX/avidin film. ( ■ ) Electrode made with the standard B-GOX/avidin, but also incorporating free biotin which blocks the avidin cross-linking. ( ● ) Electrode made using unlabeled GOX replacing B-GOX. These electrodes were prepared with the optimized 44% avidin / 56% B-GOX (GOX) ratio at constant 51μg/cm<sup>2</sup> loading. Conditions: 1000rpm, PBS pH= 7.4, air, +100mV.

Figure 14. Cathodic glucose current response for bilayer-bienzyme electrodes prepared according to the self-assembling procedure. ( □ ) 260μg/cm<sup>2</sup> avidin

immobilized electrode with B-GOX immobilized by incubation in 2mLs 100 $\mu$ g/ml B-GOX solution. ( ■ ) Identical electrode except treated with a solution of biotin before incubation with B-GOX. Conditions: 1000rpm, 20mM phosphate, pH= 7.4, air, +100mV.

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